JOURNAL OF BACTERIOLOGY, Apr. 1974, p. 149-154

Vol. 118, No. 1
Printed in U.S.A.

Active Transport of Alcohol in *Corynebacterium acetophilum*

YOSHIKATSU MUROOKA AND TOKUYA HARADA

The Institute of Scientific and Industrial Research, Osaka University, Suita, Osaka, Japan

Received for publication 15 January 1974

The transport of alcohols was studied in *Corynebacterium acetophilum*, which was isolated as a strain growing well on acetate and ethanol. The transport of ethanol was found to be inducible by ethanol, *n*-propanol, *n*-butanol, and acetate, whereas transport of methanol occurred by noninducible passive diffusion. The entry of ethanol into the cells occurred against a concentration gradient and showed saturation kinetics with two K_m values of 2.4×10^{-5} M and 6.0×10^{-5} M. Uptake of ethanol was inhibited by sodium azide, sodium cyanide, 2,4-dinitrophenol, and *p*-chloromercuribenzoate. The transport of ethanol was competitively inhibited by normal alcohols, but not by *iso*- or *tert*-alcohols. From these studies, we concluded that an inducible active alcohol transport system mediates the entry of ethanol, *n*-propanol, or *n*-butanol into the cells of *C. acetophilum*.

With the demonstration in recent years of transport systems for almost every compound of metabolic interest in bacteria, it now seems necessary to elucidate the chemical structures and molecular mechanisms involved in transport of metabolites through the limiting membrane. There have been many studies on the production of microbial cells and metabolites from alcohols (1, 7, 10, 15), but there have been none on the mechanism of transport of alcohols in bacteria. Recently we found that many strains of gram-positive bacteria and yeasts which can utilize an alcohol as a carbon source formed *O*-alkylhomoserine (8, 11, 13). With the exception of *Pseudomonas* (15), most gram-negative bacteria cannot form this compound. Therefore, it was of interest to investigate the alcohol transport system in gram-positive bacteria, in which, compared with gram-negative bacteria, relatively little is known about transport mechanisms.

This paper reports evidence of active transport of alcohols into the cells of *Corynebacterium acetophilum*.

MATERIALS AND METHODS

Bacteria and cultivation. A strain of *C. acetophilum* A51, which was isolated by Harada et al. from soil as a strain producing glutamic acid from acetic acid (9), was used in this study. Cells were grown in shaking cultures for 20 h at 30 C in 100 ml of minimal medium plus 10 mg of yeast extract and 1.0 g of carbon source. The minimal medium had the following composition: $(NH_4)_2SO_4$, 100 mg; urea, 100 mg; $MgSO_4 \cdot 7H_2O$, 10 mg; and 0.05 M phosphate buffer (pH 7.4) in 100 ml. Washed cells were regrown in the minimal medium plus a carbon source (initial concentration of cells was 6 to 10 Klett units) and harvested at the growth phase indicated. Growth was followed in a Klett colorimeter (590 to 660 nm), which was calibrated by dry-weight determination.

Alcohol transport assay. After two doublings, cells were harvested, washed twice with 0.05 M phosphate buffer (pH 7.4) at 5 C, and resuspended in the same cold buffer solution. Uptake of radioactive alcohol was measured at 30 C in 1 ml of reaction mixture containing 10^{-4} M [^{14}C]ethanol or [^{14}C]methanol, 0.05 M phosphate buffer (pH 7.4), 0.7 mg (dry weight) of cells, and 100 μg of chloramphenicol, unless otherwise stated. The mixture was shaken at 30 C for 2 min, and then [^{14}C]alcohol was added to start the reaction. The use of specific activity (0.56 Ci/mol) labeling was found to give the most sensitive and reproducible results in most experiments, even though the concentration of substrate was below the V_{max} for the uptake system. Such a procedure also mitigates the effects of diffusion which occurred at a high concentration. At intervals, cells were filtered through membrane filters (0.45 μm pore size; Toyo Roshi Co.) and quickly washed with 5 ml of the minimal medium at room temperature. Filters were dissolved in 10 ml of Bray fluid (3), and cellular radioactivity was measured with a scintillation spectrometer (Beckman LS-100). To establish the initial rate of uptake, a sample (0.5 ml) of cell suspension was rapidly pipetted into an equal volume of prewarmed medium containing the appropriate isotope or competitive inhibitor, or both. The initial rates of uptake were calculated from the linear uptakes determined at 30-s intervals for 2 min. An equal volume of cells, taken after the addition of radioactive alcohol in the presence of 0.1 mM *p*-chloromercuribenzoate and the washed cells were assayed similarly for background radioactivity. In all assays, appropriate corrections for nonspecific binding of radioactive ethanol

to *p*-chloromercuribenzoate-treated cells were made. The collection values were less than 5% of the total alcohol uptake of cells. Such corrections were particularly of importance in kinetic analysis of the transport assay.

Chromatography and autoradiography. One milliliter of the above reaction mixtures containing 0.1 μCi of [^{14}C]ethanol was separated into cells and the supernatant fractions by rapid filtration. The cells were washed and then extracted with 1 ml of ice-cold 50% acetone, and the filtered fluid and aqueous acetone-soluble fraction were lyophilized. Nonvolatile radioactive metabolites were identified by thin-layer chromatography on cellulose with the solvents *n*-butanol-acetic acid-water (4:1:5) and phenol-ethanol-water (10:7:3) (11). The aqueous acetone-soluble fraction was also reacted with 3,5-dinitrobenzoyl chloride in the presence of sodium acetate to form the nonvolatile derivatives, and the resultant alcohol derivatives were extracted by ether. The ether layer was concentrated by warming. This reaction was not disturbed with aqueous acetone and aldehyde. The yield of ethanol 3,5-dinitrobenzoate was more than 95% of a labeled ethanol standard solution. Alcohol 3,5-dinitrobenzoates were identified by thin-layer chromatography on silica gel with the solvent *n*-hexane-diethyl ether (1:1) (5). Autoradiography was performed by exposing the plates to X-ray film for 3 days. Radioactive spots were cut out, and their radioactivity was measured with a liquid scintillation counter.

Chemicals. Ethanol-*1*-^{14}*C* (57 mCi/mmol) and [^{14}C]methanol (56 mCi/mmol) were obtained from The Radiochemical Centre, Amersham Co. *O*-Ethylhomoserine was prepared in our laboratory as described previously (12). Other chemicals were of the purest grade available commercially.

RESULTS

Growth on alcohols. *C. acetophilum* was found to grow readily in a minimal medium with ethanol, *n*-propanol, sodium acetate, and glucose, but poorly with *n*-butanol and hardly at all with methanol as the sole carbon source (Table 1). The degree of growth on each carbon source corresponded with the doubling time. Growth on ethanol, *n*-propanol, or *n*-butanol after the culture had been transferred from medium with glucose or acetate was invariably preceded by a long lag period, the length of which varied with the carbon source used (unpublished data). This suggested first that the transport of alcohol was inducible, and second that the relative order of induction may be a reflection of the abilities of the cells to transport the individual alcohols.

TABLE 1. *Utilization of various compounds as a sole carbon source by Corynebacterium acetophilum*[a]

Carbon source (1%)	Dried cells produced (mg/100 ml)	Doubling time (min)
Methanol	5	
Ethanol	63	250
n-Propanol	40	400
n-Butanol	16	990
Sodium acetate	103	154
Glucose	66	240

[a] *C. acetophilum* was grown in a minimal medium plus each carbon source at 30 C. Growth was followed in a Klett colorimeter (590 to 660 nm) for 15 h, which was calibrated by dry-weight determination.

Uptake of ethanol in different media. The ability of cells to incorporate exogenous ethanol when grown on different carbon sources was determined to ascertain the induction patterns of transport. From Fig. 1, it can be seen that growth on glucose markedly repressed the uptake of [^{14}C]ethanol and that growth on alcohols induced the uptake system, with ethanol causing the most induction. Moderate uptake of ethanol was observed for cells grown on acetate. No alcohols had any effect on the uptake of [^{14}C]methanol. Ethanol uptake is enhanced

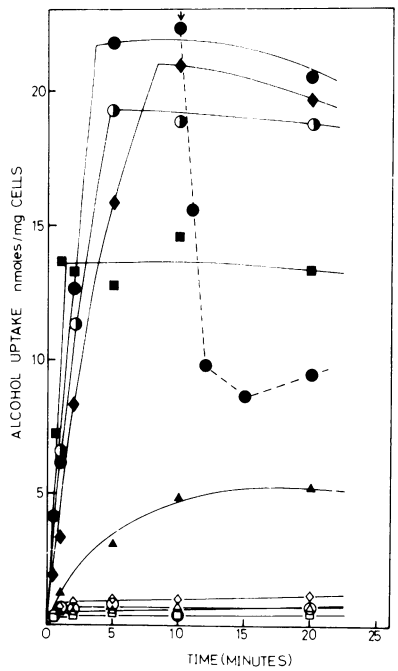

FIG. 1. *Time course of* [^{14}C]*ethanol of* [^{14}C]*methanol uptake into cells grown on various carbon sources. Closed symbols represent uptake of* [^{14}C]*methanol. The cells were grown on 1% ethanol* (○), *n-propanol* (◇), *n-butanol* (◑), *sodium acetate* (□), *or glucose* (△) *in a minimal medium. Nonradioactive ethanol (10 mM) was added at 10 min* (---●---). *Alcohol uptake was measured in the presence of 100 μg/ml of chloramphenicol.*

when cells are grown on alcohols; nevertheless, the doubling time of cells grown on alcohols was longer than that of cells grown on acetate or glucose (Table 1). Enhancement of ethanol uptake of cells grown on alcohols might be due to the inducible nature of the uptake mechanism rather than to the growth rate of the cells. When 10 mM nonradioactive ethanol was added 10 min after the addition of [^{14}C]ethanol, about 70% of the radioactivity which had accumulated in the cells was released (Fig. 1).

The intracellular concentration of ethanol was determined by thin-layer chromatography of alcohol 3,5-dinitrobenzoate of alcohol as described in Materials and Methods. A main spot of radioactivity in the thin-layer chromatography was an ethanol derivative (Fig. 2) and corresponded to 92 and 73% of total radioactivity taken up into the cells for 5 and 20 min, respectively. Furthermore, the major nonvolatile radioactive products observed by thin-layer chromatographic analysis were L-glutamic acid and *O*-ethylhomoserine, and they corresponded to about 10% and less than 1%, respectively, of the total radioactivity taken up in 60 min by cells in the presence of chloramphenicol (Fig. 3). This shows that about 90% of the radioactivity is free of volatile material. When cells in the stationary phase were exposed to 10^{-4} M [^{14}C]ethanol, 20% or more of the radioactivity was taken up by the cells in 5 min. Since the cells constitute about 0.1% of the culture volume, assuming that about 90 to 70% of the intracellular ethanol is free, this would represent a ratio of the concentrations of ethanol inside and outside the cell of about 180 to 140:1. Therefore, we concluded that ethanol was transported into the cells against a concentration gradient.

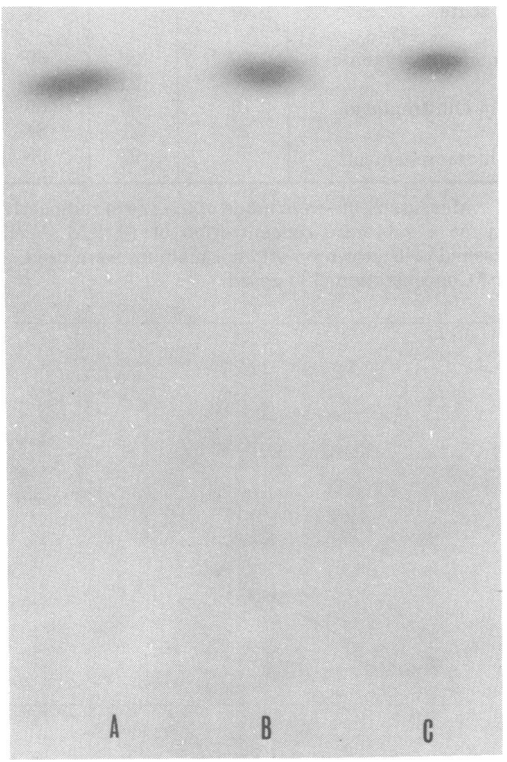

FIG. 2. *Fate of labeled ethanol in an intracellular pool. (A) 3,5-Dinitrobenzoate of [^{14}C]ethanol standard. (B and C) 3,5-Dinitrobenzoate derivatives of the extracts from cells loaded with [^{14}C]ethanol for 5 and 20 min, respectively. Autoradiograms were prepared as described in the text.*

Kinetics of transport of [^{14}C]ethanol. To determine the K_m of ethanol uptake, the initial rate of uptake was measured at different concentrations of ethanol. The ethanol transport system obeys saturation kinetics: the K_m values were calculated as 2.4×10^{-5} and 6.0×10^{-5} M (Fig. 4). However, methanol did not obey saturation kinetics.

Inhibition of ethanol transport. To gain information on the nature of the transport mechanism(s), a variety of competitive and metabolic inhibitors were tested as inhibitors of alcohol transport. *n*-Propanol, sodium acetate, and *n*-butanol were strong inhibitors of ethanol transport, whereas methanol, *iso*-propanol, *iso*-butanol, and *tert*-butanol were not found to be

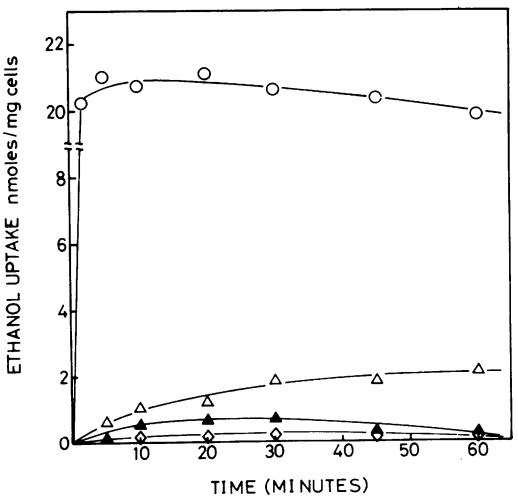

FIG. 3. *Time course of change in nonvolatile radioactive substrates in the intracellular pool and filtered fluid. Radioactive pools were established from [^{14}C]-ethanol. Ethanol uptake into the cells, ○; glutamic acid accumulated in the intracellular fluid, △; or in the filtered fluid, ▲; O-ethylhomoserine accumulated in the intracellular plus filtered fluid, ◇.*

competitive inhibitors (Table 2). Glucose had little inhibition on ethanol transport. The potent energy uncouplers, 2,4-dinitrophenol, potassium cyanide, and sodium azide, were effective inhibitors (Table 3). Monoidodoacetate, a sulfhydryl reagent, was also found not to be a strong inhibitor, whereas *p*-chloromercuribenzoate was strikingly inhibitory. This suggests that sulfhydryl groups are involved in ethanol transport. On the contrary, neither competitive nor metabolic inhibitors inhibited methanol transport.

Further kinetic studies showed that the above substrate inhibitions were competitive rather than noncompetitive. The inhibitory effects of *n*-propanol and *n*-butanol on the transport of labeled ethanol were competitive, whereas those of methanol and sodium acetate were noncompetitive (Fig. 5). From the results in Fig. 5, the K_i values of *n*-propanol and *n*-butanol were calculated to be 6.4×10^{-5} and 7.2×10^{-5} M, respectively. From this and the induction experiments, it was concluded that two transport systems are operative for alcohols in *C. acetophilum:* one is an active alcohol transport system in which the relative orders of affinity are ethanol > *n*-propanol > *n*-butanol, and the other is passive diffusion, which operates for methanol.

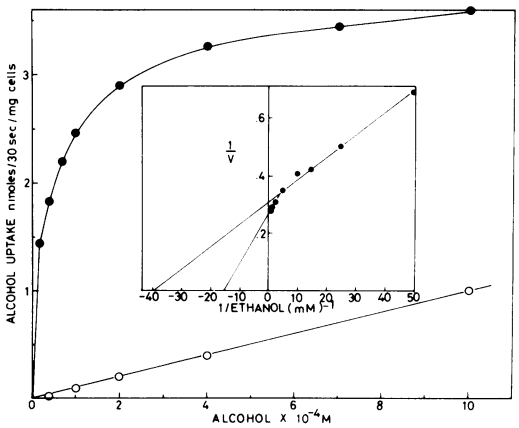

FIG. 4. *Kinetics of ethanol uptake into the cells.*

TABLE 2. *Effects of various substrates on ethanol and methanol uptakes in Corynebacterium acetophilum*[a]

Substrate added (10^{-4} M)	Relative activity (%)	
	Ethanol	Methanol
None	100	100
D-Glucose	82	102
Sodium acetate	66	91
Methanol	89	45
Ethanol	43	84
n-Propanol	56	86
iso-Propanol	95	94
n-Butanol	69	117
iso-Butanol	98	89
tert-Butanol	101	90

[a] Measurements were made of uptake of radioactivity at a substrate concentration of 10^{-4} M. Well-washed cells grown on ethanol medium were used.

TABLE 3. *Effect of metabolic inhibitors on alcohol uptake in Corynebacterium acetophilum*[a]

Inhibitor added	Concn (M)	Relative activity (%)	
		Ethanol	Methanol
None		100	100
Sodium fluoride	10^{-4}	82	95
	10^{-3}	78	96
Sodium azide	10^{-4}	36	87
	10^{-3}	29	85
Monoiodoacetate	10^{-4}	71	82
	10^{-3}	64	85
p-Chloromercuribenzoate	10^{-5}	11	83
	10^{-4}	2	71
Potassium cyanide	10^{-4}	29	60
	10^{-3}	23	67
2,4-Dinitrophenol	10^{-4}	60	86
	10^{-3}	21	84
Chloramphenicol[b]		97	95

[a] Measurements were made of uptake of radioactivity at a substrate concentration of 10^{-4} M. Well-washed cells grown on ethanol medium were used.

[b] Concentration, 100 μg/ml.

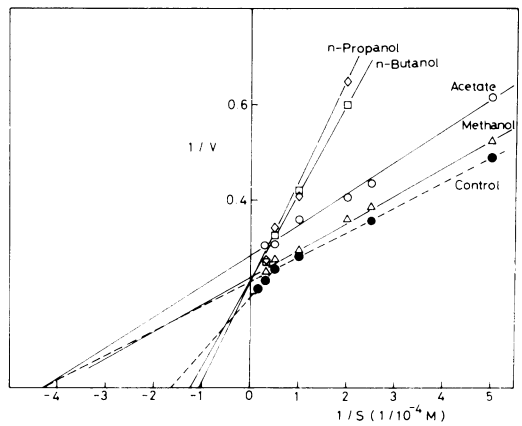

FIG. 5. *Competitive inhibition of ethanol uptake by alcohols or acetate. Alcohols and acetate were used at a concentration of* 2×10^{-4} *M. The amount of ethanol taken up at 30 C in 30 s was determined. The velocity (V) is expressed as nmol* $\times$ *30* s^{-1} *mg (dry weight)*$^{-1}$. *S is the concentration of ethanol.*

DISCUSSION

The evidence presented in this paper indicates that *Corynebacterium acetophilum* has an active transport system which is specific for normal alcohols. This transport system is required for growth with *n*-alcohols as the sole carbon source. The transport of methanol occurs by another system, namely, noninducible passive diffusion. Like the dicarboxylic acid transport system of *Bacillus subtilis* (5), the ethanol transport system here is strongly repressed by D-glucose and is strongly induced by ethanol, *n*-propanol, and *n*-butanol. These findings suggest that all *n*-alcohols act as physiological inducers of the alcohol transport system. The kinetic experiments presented in Fig. 4 show two sets of K_m values derived from curvilinear plots like those reported for amino acids transport in *Escherichia coli* (2) or *Saccharomyces cerevisiae* (6). These results suggest either that ethanol is taken up through a single carrier system which alters in affinity with change in the ethanol concentration, or that there are two distinct carrier systems, one with weak and one with strong affinity for ethanol. Conclusions on this must await genetic investigations.

From the result of the nonvolatile alcohol derivative (Fig. 2), about 90 to 70% of total radioactivity taken up into the cells for 5 to 20 min is free within the cells. Furthermore, about 70% of the [^{14}C]ethanol accumulated by this organism in ethanol broth is rapidly exchanged with nonradioactive ethanol when cells are resuspended in fresh medium or when the ethanol concentration is raised to 10 mM at 10-min incubation times (Fig. 1). This ethanol is either free within the cells or bound to proteins from which it can dissociate rapidly. The remaining 30% of the ethanol is not exchangeable, and about 10% was detected as L-glutamic acid even for 60 min (Fig. 3). Since ethanol transport is a requisite for growth on *n*-alcohol, it would be hard to obtain the truly alcohol-free conditions. Nevertheless, cells can concentrate ethanol more than 180-fold from media containing a trace amount of [^{14}C]ethanol.

When dealing with energy-dependent uptake, the question always arises of whether the requirement occurs at the level of intracellular accumulation. Results on metabolic inhibition of ethanol uptake seem to confirm the requirement for energy in this transport system. Analogues may well be toxic to various enzymes. However, ethanol uptake is not inhibited by methanol, *iso*-propanol, *iso*-butanol, or *tert*-butanol. The effect of acetate seemed to be similar to that of *n*-alcohols. However, kinetic analysis showed that the inhibition was noncompetitive. This suggests that the transport of acetate occurs by another system and that inhibition by acetate might be similar to that by 2,4-dinitrophenol, as reported for amino acids transport in *B. subtilis* (16). These possibilities are currently under investigation.

The uptake of methanol is quite different from that of ethanol. From induction, kinetic, and inhibition experiments, we concluded that uptake of methanol occurs by noninducible passive diffusion. Peculiarly, *C. acetophilum* is unable to use methanol readily as a carbon source. From the kinetic analysis described in this paper, methanol apparently enters the cells. Therefore, this organism probably lacks methanol-assimilative enzymes.

LITERATURE CITED

1. Abbott, B. J., A. I. Laskin, and C. J. McCoy. 1973. Growth of *Acinetobacter calcoaceticus* on ethanol. Appl. Microbiol. **25**:787-792.
2. Anraku, Y., T. Naraki, and S. Kanzaki. 1973. Transport of sugars and amino acids in bacteria. VI. Changes induced by valine in the branched chain amino acid transport systems of *Escherichia coli*. J. Biochem. (Tokyo) **73**:1149-1161.
3. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
4. Cheronis, N. D., and J. B. Entrikin. 1965. Semimicro qualitative organic analysis: derivatives of alcohols and phenols 3,5-dinitrobenzoates. John Wiley and Sons, Inc., New York.
5. Ghei, O. K., and W. W. Kay. 1973. Properties of an inducible C_4-dicarboxylic acid transport system in *Bacillus subtilis*. J. Bacteriol. **114**:65-79.
6. Gifts, J. J., and M. Grenson. 1967. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. III. Evidence for a specific methionine-transporting system. Biochim. Biophys. Acta **135**:507-516.
7. Harada, T., and T. Hirabayashi. 1968. Utilization of alcohols by *Hansenula miso*. Agr. Biol. Chem. **32**:1175-1180.
8. Harada, T., and M. Okuhara. 1971. Formation of *O*-alkylhomoserines from alcohols, by yeast capable of assimilating 1,2-propandiol. Agr. Biol. Chem. **35**:126-127.
9. Harada, T., K. Seto, and Y. Murooka. 1969. Formation of glutamic acid from acetic acid by *Corynebacterium acetophilum* nov. sp. J. Ferment. Technol. **46**:169-176.
10. Mor, J. R., and A. Fiechter. 1968. Continuous cultivation of *Saccharomyces cerevisiae*. I. Growth on ethanol under steady-state conditions. Biotechnol. Bioeng. **10**:159-176.
11. Murooka, Y., and T. Harada. 1968. Formation of *O*-ethylhomoserine by bacteria. J. Bacteriol. **96**:314-317.
12. Murooka, Y., T. Harada, and Y. Izumi. 1968. Chemical synthesis of *O*-ethyl-L-homoserine, a new amino acid produced by *Corynebacterium ethanolaminophilum*. Bull. Chem. Soc. Japan **41**:633-635.
13. Murooka, Y., K. Seto, and T. Harada. 1970. *O*-Alkylhomoserine synthesis from *O*-acethylhomoserine and alcohol. Biochem. Biophys. Res. Commun. **41**:407-414.

14. Oki, T., Y. Nishimura, Y. Sayama, A. Kitai, and A. Ozaki. 1969. Microbial utilization of non-carbohydrate substances. I. Fermentative production of glutamic acid from ethanol. Amino Acid Nucl. Acid (Tokyo) **19**:73–81.
15. Oki, T., Y. Sayama, Y. Nishimura, and A. Ozaki. 1968. L-Glutamic acid formation by microorganisms from ethanol. Agr. Biol. Chem. **32**:119–120.
16. Sheu, C. W., W. N. Konings, and E. Freese. 1972. Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. J. Bacteriol. **111**:525–530.